

The University of Chicago

THE OXIDATION-REDUCTION
REQUIREMENTS OF A NON-SPORE
FORMING OBLIGATE ANAEROBE

A PART OF A DISSERTATION SUBMITTED TO
THE FACULTY OF THE DIVISION OF THE
BIOLOGICAL SCIENCES IN CANDIDACY FOR
THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF BIOCHEMISTRY

1938

By
BIRGIT VENNESLAND

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Reprinted from
THE JOURNAL OF BACTERIOLOGY, Vol. 39, No. 2, February, 1940



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THE OXIDATION-REDUCTION POTENTIAL REQUIREMENTS OF A NON-SPORE-FORMING, OBLIGATE ANAEROBE^{1, 2}

BIRGIT VENNESLAND

INTRODUCTION

The specification of the reducing conditions which determine the growth of obligate anaerobes has been the object of many investigations. Early observers stressed the maximum oxygen pressure under which the organisms would grow (Clark, 1924; McLeod, 1931) as well as the enhancement of growth by the addition of reducing substances to the medium (Quastel and Stephenson, 1926; Wurmser, 1930). Then, after the theory of reversible oxidation-reduction potentials had been developed (Cannan, Cohen, and Clark, 1926), several attempts were made to determine in the medium a limiting potential value above which anaerobes were unable to multiply. Aubel, Aubertin, and Genevois (1929), working with eight different spore-forming anaerobes and measuring potentials with dyes, concluded that an rH above twelve (E_h of -0.060 v. at pH 7) was incompatible with anaerobic growth. Plotz and Geloso (1930) working with electrodes and dyes, established for *Clostridium tetani* a maximum oxidation-reduction potential of $+0.036$ v. at pH 7.0 (rH of 15). Knight and Fildes (1930) poisoning their potentials with a N_2 , O_2 mixture, found positive limit of $+0.110$ v. at pH 7.2 (rH of 17) for *Clostridium tetani*. Knaysi and Dutky (1936) worked with a butanol-producing *Clostridium* and attempted to separate the two

¹ A preliminary report of this material was given at the Toronto meeting of the American Society of Biological Chemists, April 1939.

² These investigations were supported in part by a grant from the Rockefeller Foundation.

factors, oxygen tension and potential, by poisoning their medium with potassium ferricyanide. They concluded that high potentials induced by oxygen were more efficacious in limiting growth than high potentials maintained by ferricyanide. On the other hand, Kligler and Guggenheim (1937) working with *Clostridium welchii* concluded that enhancement of growth brought about by vitamin C was related to the lowering of the potential and not to the absorption of the oxygen in the medium. They measured the potential colorimetrically with indigo carmin and established a limit of -0.125 v. at pH 7.2.

The wide discrepancies among the results of various workers indicate that further investigation of the problem may be of value. In the following study an attempt has been made to determine that potential which just inhibits the growth of a strain of *Bacteroides vulgatus*, a different type of anaerobe from those previously studied.

EXPERIMENTAL

A culture of *Bacteroides vulgatus*, strain Marino, was kindly furnished us by Dr. A. H. Eggerth of the Long Island College of Medicine. This organism is a commonly occurring, gram-negative, non-spore forming anaerobe of intestinal origin (Eggerth and Gagnon, 1933; Weiss and Rettger, 1937). It was maintained with frequent transfers on a buffered, glucose brain-broth medium.

The experiments were conducted with the purpose of establishing the most positive potential level at which this organism would grow on a glucose, nutrient broth medium. Measurements of pH were made with glass electrodes, of potentials with shiny platinum electrodes, against a saturated calomel cell, all of which were set up in an incubating room kept at 38°C. In the first experiments, measurements were made with a Leeds and Northrup portable potentiometer-electrometer (cat. no. 7660). Later, a Hellige vacuum-tube galvanometer attached to an ordinary potentiometer was used. When this latter instrument was employed, all electrodes were enclosed in a copper shield. A resistance of twenty megohms was always inserted in the platinum electrodes circuit to prevent polarization.

The glass electrodes were of a design used with success in this laboratory for several years. A small bead of Corning 015 glass is fused on to the tapered end of an ordinary soft glass tube and blown out into a small, relatively thin bulb. A phosphate buffer solution approximately M/20 in both iodine and potassium iodide (exact concentrations unimportant) is filled into the tube. A thin platinum wire is sealed into the open end of the tube so that one end dips into the solution and the other protrudes outside.

These glass electrodes were found to be much more sensitive at 38°C. than at room temperature. They were calibrated at 38°C. with M/10 HCl and a phosphate buffer of known pH immediately before and after each experiment. Significant changes in calibration during the course of two or three days were seldom observed. The maximum error in the pH determinations was judged to be 0.05 pH, while the sensitivity was 0.02 pH. The accuracy of the platinum electrode readings will be discussed in a later section.

Experiments with mixed cultures

In the first series of experiments the necessary reducing conditions were established by growing a non-fermenting obligate aerobe, *Alcaligenes fecalis* with the *Bacteroides vulgatus*. It was thought that the potential at which acid production just starts should indicate the maximum potential compatible with growth of the *Bacteroides* since the aerobe ferments no sugars, while the anaerobe characteristically forms acid when growing on a glucose medium. Although these experiments with mixed cultures did not successfully define a limiting potential for anaerobic growth, as the later experiments in pure cultures did, they are none the less described here because they have a number of points of incidental interest.

Electrode vessel. For these experiments a special electrode vessel was devised (fig. 1).

An ordinary test tube ($\frac{3}{8}$ by 6 inches) is provided with three side arms, A, B, and C, as indicated. A shiny platinum electrode, D, is inserted into the lower side arm, B, and held in place by a

tightly fitting piece of rubber tubing. A vial, *E*, $\frac{7}{8}$ by 3 inches, is filled to the point indicated with three per cent agar made up with a solution of about the same salt composition as the medium which is to be used. A twohole rubber stopper provided with the glass tubes, *F* and *G*, is fitted into the vial. Then *C* is joined to *G* with a piece of rubber tubing which is closed with a screw clamp, *H*. *C* is of smaller diameter so that any change in the composition of its contents will not appreciably affect that of the rest of the tube.

The large tube is filled with the culture medium to about the level indicated. The openings *A*, *I*, and *F* are plugged with cotton and the entire assembly is autoclaved. During the auto-

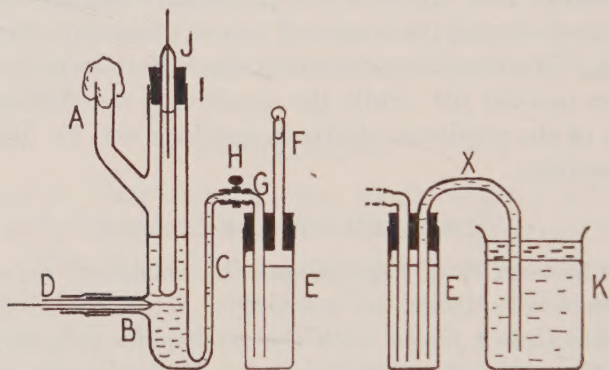


FIG. 1

claving, air is driven out of the connecting arms *C* and *G*, and the medium and the agar rises to the clamp, *H*. After the agar has solidified, electrical connection between *C* and *G* is easily established by opening *H*. Tube *F* is then removed and the remaining space in *E* is filled to the top of the opening in the rubber stopper with a saturated solution of potassium chloride. Connection with a saturated potassium chloride reservoir (*K*) is established by plunging a U-tube into the opening of the stopper as indicated in *X*. A layer of mineral oil on the open reservoir eliminates evaporation. The calomel cell is connected with the saturated KCl reservoir. In this manner a conducting pathway is easily established between the medium and the calomel cell

without any danger of contamination and with negligible diffusion of KCl from the bridge.

The side arm *A* is used for inoculations. A rubber stopper which fits the culture tube is slipped over the top end of the glass electrode. Sterilization of the glass electrode is accomplished by immersion for three minutes in 0.1 per cent mercuric chloride (Longsworth and MacInnes, 1935). The electrode is then rinsed well with a stream of sterile distilled water and inserted into *I* after the cotton plug has been removed.

This apparatus was devised particularly for use with small quantities of liquid, a desirable feature when a synthetic medium is employed. It does not, of course, provide for equilibration with a gas mixture or for any stirring.

Experiments with peptone medium. The medium used generally consisted of five grams of Bacto-Peptone (Difco) three grams Bacto Beef Extract (Difco), ten grams sodium chloride and five grams glucose in one liter of water. The pH after autoclaving ranged from 6.2 to 6.7 with an average of 6.6. No buffer was added because as rapid a pH change as possible was desired. Omission of the salt or raising of the sugar concentration to 1.0 per cent had no effect on the results. In most of the experiments inoculations were made with approximately 0.1 ml. of a light suspension of *Alcaligenes* from an agar slant and 0.1 ml. of a brain broth culture of *Bacteroides*.

Figure 2 shows the result of a typical experiment obtained with a mixed culture and with *Alcaligenes* alone. About fifteen experiments were done in all, and in general, the potential and pH curves obtained in separate experiments were quite similar. Acid production started at varying times, from about five to thirty hours after inoculation, depending mainly on the freshness of the inoculum used. It was practically complete in about twenty-five additional hours, at a final pH of 5.0 (± 0.2). Prolonged incubation showed little further change in pH. There was usually a rather sharp fall in potential just preceding the first detectable acid production. This fall continued to about the point where acid production was half complete and most rapid, E_h about -0.200 v. at pH 6, after which the potential rose again slowly (while the pH

continued to fall), reaching E_h -0.150 v. at pH 5.0, with considerable variation in the numerical values in individual cases. In tubes containing *Alcaligenes fecalis* alone, the potential gradually became more negative, reaching E_h of about zero after fifteen

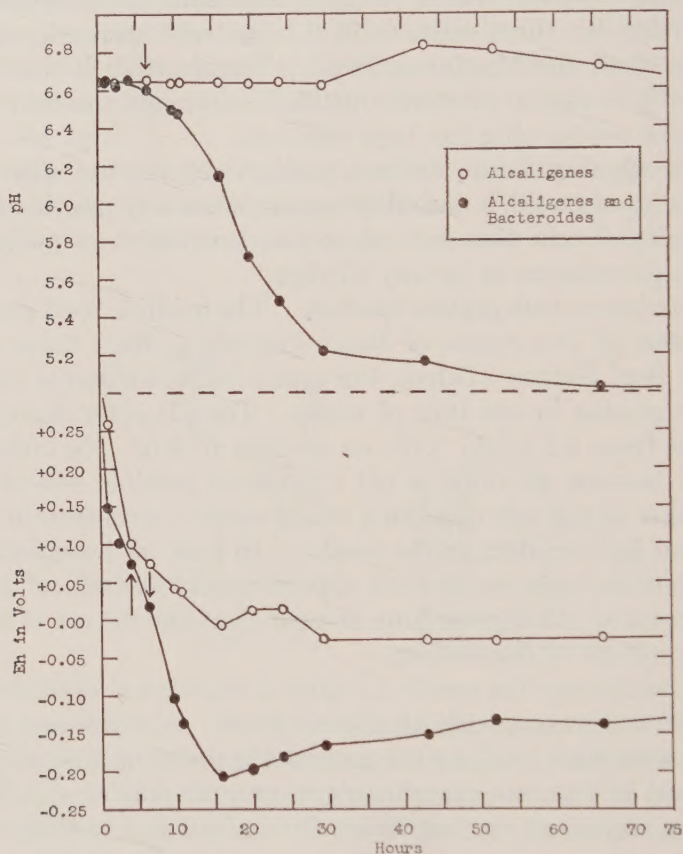


FIG. 2. pH AND POTENTIAL CURVES ON CULTURES OF *ALCALIGENES FECALIS* AND ON MIXED CULTURES OF *BACTERIOIDES VULGATUS* AND *ALCALIGENES FECALIS*

to forty-eight hours; while the pH became very slightly more alkaline by about 0.2 pH.

Uninoculated control tubes showed constant pH values for the first few days, but if incubation was continued much longer a very slight acid drift appeared. Media inoculated with small

quantities of old *Bacteroides* cultures behaved like the controls except that the acid drift was a little more marked. If a sufficiently large and fresh inoculum, for example one-tenth milliliter of a two-day brain broth culture, was used, and the medium had been freshly auto-claved, growth occurred without addition of *Alcaligenes*. Here, however, the pH curves were not smooth, pH and potential readings could be changed by stirring, and there was an obvious lack of homogeneity in the medium, greater turbidity occurring in the lower layers of the liquid.

In an attempt to define a limiting potential for acid production from these data, there was noted that potential at which acid production started. This is marked in figure 2 by downward arrows, ↓, and the numerical values for this and three other experiments are given in the second column of table 1. The wide

TABLE 1

TUBE NUMBER	POTENTIAL WHEN ACID PRODUCTION STARTED	POTENTIAL WHEN SHARP POTENTIAL DROP STARTED
	<i>E_h</i> -volts	<i>E_h</i> -volts
1	0.000	+0.055
2	-0.100	+0.080
3	+0.065	+0.065
4	+0.020	+0.075

variation here, -0.100 to +0.065 v., at once suggests that it is not very significant. Ten other experiments under the same conditions showed similar results.

Since in the mixed cultures a much greater drop in potential is observed, than is present in the cultures of *Alcaligenes* alone, the potential at which this greater drop begins should be a better measure of the limiting potential for the anaerobe, and these values are given in the third column of table 1 (indicated by an upward arrow, ↑, in figure 2). These values, +0.055 to +0.080 v., show better agreement, but they are also not very significant; first, because they are chosen arbitrarily from the graphs; and secondly, because they represent readings at a time when the potential is changing rapidly, and large errors may arise

from variable sensitivities among the electrodes in registering potential changes in solution.

It is apparent that a different method is necessary to characterize accurately the limiting potential, although it seems from these experiments that the limit is probably above $E_h + 0.050$ v. The method employed, however, is particularly faulty in that it does not provide for the maintenance, for a considerable period of time, of high and constant potential levels.

Rates of acid production. As a matter of incidental interest, titration curves were obtained for several batches of medium used; and the rate of acid production was calculated from these curves in terms of milliequivalents of strong acid per liter per hour in the range of maximum rate of acid production (pH 6.2 to 5.8).

This was also done with *Bacterium coli*, and *Proteus vulgaris*. The media were the same, five grams peptone, three grams beef extract, and five grams glucose in one liter, except that in the case of *Bacteroides*, five grams sodium chloride per liter was added.

It required one cubic centimeter of normal acid per liter to cause a pH change from 6.32 to 5.88 with this medium, which means a buffer value of 2.3 milliequivalents. The maximum pH changes per hour observed in the actively growing cultures for *Bacterium coli*, *Proteus vulgaris*, and *Bacteroides vulgatus*, are respectively 1.41, .72, and .104 and the corresponding milliequivalents of acid per liter per hour are 3.20, 1.63, and 0.24. It will be noted that *Bacteroides vulgatus* produces acid much more slowly under the observed conditions than do the other two organisms.

Experiments with synthetic medium. A synthetic medium of the following composition (formula furnished by Dr. A. H. Eggerth) was used for a few experiments: water, 200 ml.; asparagine, 0.5 gram; Histidine dihydrochloride, 0.2 gram; Cysteine hydrochloride, 0.2 gram; $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 0.5 gram; KCl, 0.2 gram; MgSO_4 , 0.05 gram; glucose, 2 grams; adjusted to pH 7.6.

The results are summarized in figure 3, where pH, E_h , and the corresponding rH values are plotted against time. In comparing the *Alcaligenes* culture with the mixed culture of *Bacteroides* and *Alcaligenes*, it may be noted that although the pH and potential values are widely different, the final rH values are the same.

The anaerobe lowers the potential more rapidly and extensively during the fermentation. A double reversal of potential similar to that observed in acid-producing cultures (Hewitt, 1936, page 67) of various organisms is evident here. The rH values show that it is not attributable to the pH changes. It does, how-

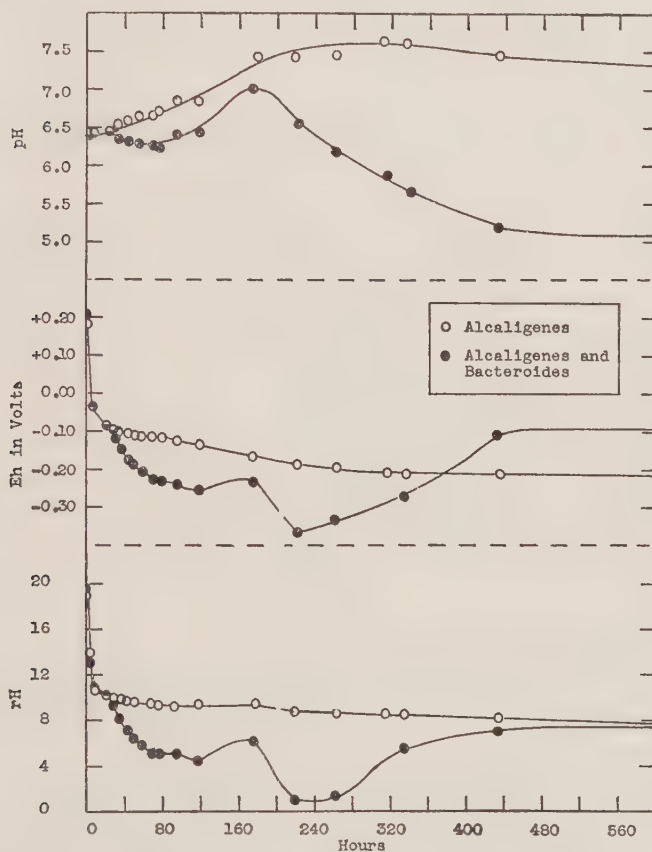


FIG. 3. pH, POTENTIAL, AND rH CURVES OF ALCALIGENES AND OF BACTERIODES AND ALCALIGENES GROWN ON A SYNTHETIC MEDIUM

ever, seem to depend on a change from alkali production to acid production.

A sterile control (not graphed) had a constant pH of 6.4. The potential was +0.200 v. at the beginning of the experiment and showed a positive drift, reaching +0.330 v. at the end.

Rates of acid production on the synthetic medium varied from 0.035 to 0.045 milliequivalents per liter per hour, and are seen to be considerably lower than those on the nutrient broth medium.

Because of the complexity of the factors involved in the pH changes, it is impossible to determine the point at which acid production by the anaerobe begins in the mixed cultures, and so it is impossible to determine any limiting potential for growth from these data.

Experiments with pure cultures of Bacteroides and gaseous control of anaerobic conditions

Our previous experiments had demonstrated the need for a procedure by which potential levels could be maintained constant for a chosen period of time. Knight's (1930) method of poisoning potentials by means of a mixed stream of nitrogen and oxygen seemed to be the best means of maintaining a definite potential level under conditions not otherwise injurious to growth. This method was therefore adopted.

The gas mixture was prepared as outlined by Knight. Nitrogen, purified by passage over glowing copper, was mixed with unpurified nitrogen (by-passed from the same tank) in the proportions necessary to maintain a certain potential level. An additional inlet tube served to admit air when necessary. Three outlet tubes for the gas mixture were arranged in parallel so that three similar experiments could be run at the same time. The medium used was the same as that employed in the previous experiments, except that ten grams of glucose were added per liter instead of five.

Electrode vessel. The electrode vessels were set up as shown diagrammatically in figure 4. *A* is a four-ounce, wide-mouth bottle into which eighty milliliters of the medium are measured. *B* is an inlet tube for the gas mixture. It is constricted at the lower end so that the entering gas may be broken up into a stream of small bubbles. An estimated volume of fifteen milliliters of gas passed through the culture in a minute. *C* is an inlet tube for inoculation and serves at the same time as an outlet for the gas. *B* and *C* are both plugged with cotton. Bulbs are blown

in these tubes to keep the medium from wetting the cotton plugs during the autoclaving. *D* is a glass tube to hold the glass electrode. It is plugged with cotton during the autoclaving. *E* is a capillary tube joined to an agar container. *F* and *G* are shiny platinum electrodes,³ platinum wires, 0.75 mm. in diameter and 2 cm. in length. In some experiments three platinum electrodes were used.

In studying the effect of area of platinum on the readings it was found that large foil electrodes (area about 4 square centimeters) gave no better results than the small wire ones. The electrodes

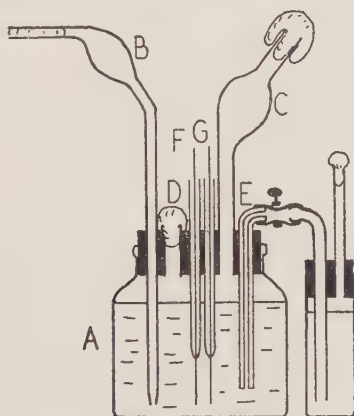


FIG. 4

were cleaned by heating to glowing in a hot flame before use. This procedure usually necessitated resealing at the same time.

³ A rather high degree of variability was observed in the agreement obtainable between two electrodes in the same solution. In general, electrodes agreed within ten millivolts except when the potentials were high (above 0.200 v.) or were changing rapidly. Agreement almost always improved as the potential went down (to zero and below), indicating apparently better poisoning at the lower levels. Discrepancies between electrodes could often be ascribed only to inherent differences in the electrodes themselves. In one extreme case two electrodes which were apparently all right consistently gave readings about fifty millivolts apart at the higher potential levels (+0.100 to +0.200 v.), while they differed by about 0.005 v. at E_h of 0. It is evident that very good checks may indicate nothing more than a fortuitous similarity between the electrodes chosen. The characterization of levels closer than ten millivolts, the ordinary range of agreement, consequently has no significance.

All electrodes were checked before and after each experiment in solutions of known, well-poised potential. Only those electrodes which gave correct readings in such solutions were used. If an electrode did not give correct readings when tested at the termination of an experiment, all results obtained with it were discarded. (In such cases it was usually found that the electrode had been cracked during the autoclaving.) Unless one of the electrodes was discarded, the potentials listed are averages of two or three electrode readings.

The electrode vessel is autoclaved with medium and platinum electrodes. Connection with the calomel half cell is made as described in the previous section. The glass electrodes are also prepared and inserted as described before. Instead of a rubber stopper, however, a piece of thin rubber tubing slipped over the upper end of the glass electrode serves to hold it in place in the wider tube, *D*.

Necessity for CO₂. The first attempts to grow *Bacteroides vulgatus* by this procedure were completely unsuccessful. For example, an inoculum of two milliliters of a two-day brain broth culture showed no growth although the potential was kept below an E_h of zero for forty hours at a time. The medium used gave good growth in twenty-four hours when incubation occurred over pyrogallol and carbonate in an anaerobic jar. Then it was found that if the gas mixture was passed through a saturated solution of sodium bicarbonate before being admitted to the culture flasks, growth occurred very readily. Apparently CO₂ is essential for the organism and the nitrogen sweeps this necessary gas out of the medium. A total of eleven experiments without CO₂ were attempted and signs of growth were never observed. On three occasions two experiments were run side by side in an identical manner except that the nitrogen was bubbled through water in one and through a saturated bicarbonate solution in the other. *Bacteroides vulgatus* grew in those cases where CO₂ was furnished; and never in its absence. Furthermore, in all subsequent experiments, failure of growth was never observed when CO₂ was furnished and the potential was sufficiently low.

Table 2 shows the average values obtained for pH's and CO₂-

contents of some batches of uninoculated media treated according to the procedure usually followed in the experiments. The medium was adjusted to a pH of 7.4 before autoclaving so that it would bind a greater amount of CO₂. The pH dropped to 7.2 during the autoclaving, and the addition of CO₂ brought it down to about 6.6, the final level attained depending partly on the rate at which gas was passed through the culture. This was thought to be due to the fact that the gas was probably not in full equilibrium with the bicarbonate; and a change of rate of gas flow changed the degree of equilibration achieved. The carbon dioxide contents of the media in most of our experiments fell in the range shown in the table. Although no attempt was

TABLE 2
CO₂ determination on medium before and after the passage of nitrogen

TREATMENT	pH	CO ₂ mM/liter
No gas.....	7.26	0.14
N ₂ through water for 1½ hours.....	7.28	0.08*
N ₂ through saturated bicarbonate solution for 2 hours at rate of 12 ml. per minute.....	6.55	3.03
N ₂ through saturated bicarbonate solution for 2 hours at rate of 18 ml. per minute.....	6.63	2.38

* This value might have fallen still lower if the gas flow had continued for more than 1½ hours.

made to determine the minimum amount of CO₂ required, a few experiments indicated that addition of extra free base to the medium was unnecessary. The values listed for CO₂-contents are therefore well above the minimum requirements of the organism.

In demonstrating the need of *Bacteroides vulgatus* for CO₂, we have only added another name to the long list of organisms which are already known to require this gas (Gladstone, Fildes, and Richardson, 1935). Besides the fact that CO₂ is so widely required, there is other evidence to indicate that, even for heterotrophic organisms, it is more than an inert end-product, of importance mainly because of its pH effects. Hes (1938) has indicated,

in a preliminary report, that the removal of CO_2 completely inhibits certain methylene blue reductions effected by some substrates in the presence of cell suspensions of *Bacterium coli* and of *Bacterium prodigiosum*.

Determination of the limiting potential—Procedure. The following procedure was finally adopted in order to determine the potential which just inhibited growth of the organism. Three culture vessels were set up in an exactly similar manner. The nitrogen was led off through three outlet tubes into three wash bottles containing saturated sodium bicarbonate solution, each of which was connected with a culture vessel. An extra inlet tube in each wash bottle was connected with an air line so that air could be admitted independently into each culture vessel. The gas inlet tubes in the wash bottles had to be of large internal diameter; otherwise the solid formed by evaporation would occlude the tube. The approximate proportions of air and nitrogen used were estimated by counting bubbles in the bicarbonate containers. In a set of three simultaneous experiments, one was run as an inoculated control, in which the potential was kept sufficiently negative to insure uninhibited growth; the other two were maintained at definite, more positive potential levels. Purified nitrogen was first bubbled rapidly through all the flasks in order to bring the potential down to the desired level rapidly. At the same time the pH in each flask changed from about 7.2 to 6.6 because of the addition of CO_2 (see table 2). Then the composition of the gas mixtures in the second and third vessels was regulated by the controlled addition of oxygen in order to maintain the desired potential level. Large, fresh inocula (0.5 to 1.0 ml. 2-day culture) were used in order to decrease the lag period, and these made it necessary to add air to the nitrogen rather soon in order to maintain the more positive potential levels. Although potentials of sterile media remain constant when a constant gas mixture is used, the inoculated medium shows an immediate lowering of potential which increases with continued incubation even though the same gas continues to bubble through the culture (provided the inoculum is not too small or the original potential too high). Part of this initial lowering is undoubtedly

due to the reducing substances introduced with the inoculum, rather than to growth of the organism.

It was possible to maintain the potential of the inoculated medium at a given level, only by frequent readjustment of the composition of the gas mixture, and when growth was occurring this involved a progressive increase in the proportion of O_2 . Readings were taken every fifteen minutes or oftener and numerous changes made in the rate of flow of the air in order to keep the potentials even approximately constant. Some fluctuation in the potential levels was unavoidable.

The inoculated control was allowed to attain growth as rapidly as possible, that is, only nitrogen and carbon dioxide, no air, was bubbled through. The other two vessels were then compared with the control in order to determine whether there had been any inhibition of growth. The pH level, after the first big drop, stayed at a relatively constant value, but because of the possibility of changes in the carbon dioxide tension, small pH changes, less than 0.05 pH, were not regarded as significant. Conclusions could be based only on steady continued changes.

Criteria for growth. Since no plate counts were made in the course of these experiments, some justification is required for the frequent use of the expression "growth" in describing the nature of the results. This term has been used to designate a group of phenomena all of which are believed to be associated with an actual increase in bacterial protoplasm. They may be listed as follows: (1) increase in reducing power, (2) initiation of acid production, and (3) increase in turbidity. By increase in reducing power is meant the increase in the proportion of oxygen necessary to maintain a given potential in a culture over a period of time after inoculation. This phenomenon will be discussed in more detail in the later description of the results. The other terms are self-explanatory. Under the experimental conditions employed, the three phenomena mentioned above could be dissociated only in time. If the first one appeared under given conditions, the others invariably followed, provided the same conditions were maintained for several hours. The appearance of increase in reducing power, then, was evidence that initiation of

acid production and increase in turbidity would eventually occur. Increased turbidity itself needs no justification as a criterion for growth. However, since the inoculum usually imparted some turbidity to the medium to start with, the increase was not quickly apparent and its grades of intensity were difficult to judge. A more quantitative and sensitive measure of activity in the culture was furnished by the increase in reducing power and by the change in pH; and since these always eventually led to an increase in turbidity, their appearance will be referred to as growth. It should be understood, however, that no claims are made about the time when actual number increase begins, nor is it thought that this point is particularly significant.

The use of acid production as a criterion for growth is, of course, open to objection. It is recognized that fermentation can occur under conditions which preclude any increase in bacterial protoplasm. An appreciable rate of acid production is, however, dependent on the presence of a fairly large concentration of organisms, and the original inocula which were used were not thought to be large enough to cause a marked rate of pH change without a preliminary increase in the number of organisms present. However, it is possible to use the other two criteria mentioned, exclusive of any pH change, and come to the same conclusions about the location of the limiting potential. Increase in reducing power and appearance of turbidity at any potential level were always accompanied by the establishment of an appreciable rate of acid production. Consequently, if there are separate potential limits for growth and acid production, the limit for acid production is not lower than, but equal to or higher than the limit for protoplasmic increase. Failure of appearance of acid production at high levels in our experiments may be due to a specific effect of the high potentials on the fermentative mechanism, as well as to retardation of growth. Since the organism can grow without glucose, it is impossible from our data to evaluate the relative importance of these two possibilities. The relationship of this aspect of the problem to the Pasteur phenomenon is obvious (see also Chaix and Fromageot, 1939); but it has not been our purpose to investigate this particular point.

Results. Figure 5 gives complete data obtained in one typical experiment. pH's, potentials, and fractional volume of air are all plotted on the same sheet for each of the three cultures: *A* is the inoculated uninhibited control, *B* is kept at an average potential of $+0.150$ v. for ten hours, *C* is kept at an average of $+0.108$ v. for six hours.

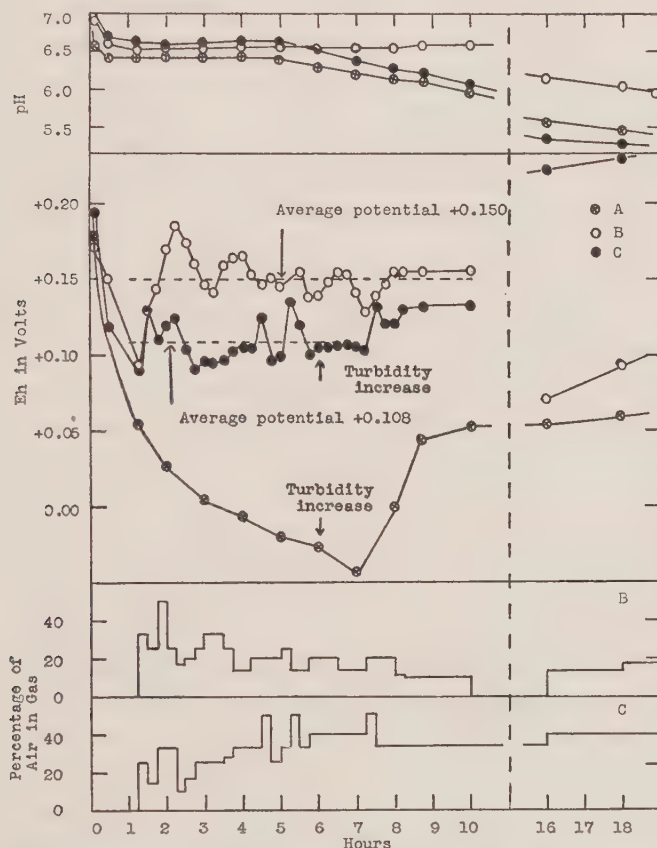


FIG. 5. pH AND POTENTIALS OF BACTERIODES CULTURES KEPT AT CONTROLLED POTENTIAL LEVELS BY A NITROGEN-AIR GAS MIXTURE

It is seen from *B* that a potential level of $+0.150$ v. at a pH of about 6.6 was unaccompanied by acid production, appearance of turbidity, or any marked increase in reducing power. That the culture was apparently uninjured at this level, however, was

shown by the appearance of all these phenomena when the air was subsequently shut off for six hours. The culture, *C*, kept at a level of +0.108 v. at pH 6.7, on the other hand, showed acid production and turbidity on a level with that in the control, or even greater.

The dependence of the potential upon the proportion of air in the gas mixture is readily seen. The fluctuating potential level, of course, combines with the uncertainty of the electrode readings in making it impossible to determine a limit more precisely than 10 millivolts. The average deviation of individual potential readings for the average potential level was found by calculation to be 0.0092 v. in *B* and 0.0095 v. in *C*. The proportion of air in the gas mixture was measured very roughly, and changes occurred much more frequently than could be recorded, but comparative values are significant and the graphs show how in *C*, during growth (average EMF +0.108 v.), the reducing power gradually increases, while in *B*, with no growth (average EMF +0.150 v.), it remains relatively constant, or shows a slight decrease. In other words, it was found that when the organism was growing (as indicated by definite acid production and eventual turbidity), progressively increasing amounts of oxygen had to be admitted in the gas mixture in order to keep the potential at a constant level; and so, near the end of an experiment with a growing culture, the organism can tolerate and grow at an oxygen tension which at the beginning would have precluded all growth.

At the end of the sixth hour the furnace which heated the copper used to purify the nitrogen was shut off; so that shortly thereafter the control vessel *A*, which had previously been receiving mainly purified nitrogen, was receiving unpurified nitrogen. The rise in potential up to 0.050 v. which accompanied the addition of oxygen contained in the impure nitrogen is clearly shown on the graph. It is important to note, however, that in other experiments where small, old inocula were used, the use of impure nitrogen from the beginning of the experiment maintained the potential at a sufficiently high level to prevent all growth.

These observations show that the oxygen tension which the organism can tolerate varies over very wide limits with the state

of the culture, and that it is not possible in general to define a particular oxygen tension which will limit growth of the anaerobe. The potentials in the control tube cannot be considered characteristic of the organism, since no precautions were taken to exclude all traces of oxygen.

TABLE 3

EXPERIMENT	DURATION	POTENTIAL		TIME IN WHICH ACID PRODUCTION BEGAN	pH WHEN ACID PRODUCTION BEGAN	RATE OF pH CHANGE	INCREASE IN REDUCING POWER	PER CENT AIR IN GAS MIXTURE AT END OF EXPERIMENT
		Average E_h level	Average deviation from average E_h level					
	hours	volts	volts	hours		ΔpH per hour		
1 A*	10	ca. 0		5	6.40	0.10		
1 B	10	+0.150	0.009		6.55	0.00	—	10
1 C	10	+0.108	0.009	5	6.65	0.12	+	30
2 A*	10	ca. -0.030		4	6.50	0.08		
2 B	10	+0.123	0.003	4	6.42	0.04	+	30
2 C	10	+0.104	0.006	4	6.42	0.04	+	25
3 A*	11	ca. +0.040		5	6.66	0.08		
3 B	11	+0.125	0.009	5	6.70	0.06	+	30
3 C	11	+0.132	0.008	5	6.75	0.03	+	15
4 A*	12	ca. 0		6	6.55	0.06		
4 B	12	+0.151	0.006	8	6.51	0.03	+	10
4 C	12	+0.142	0.004	8	6.56	0.03	+	15
5 A*	15	ca. 0		6	6.65	0.11		
5 B	15	+0.158	0.008			0.00	—	3
5 C	15	+0.153	0.009	10	6.63	0.05	+	30

* Control.

In table 3 the data of the foregoing experiment are summarized, together with those of several additional experiments conducted at intermediate potential levels. It is apparent that growth occurs readily at E_h levels averaging +0.132 v. and below: 1 B, at a potential of +0.150 showed no growth; 4 B, at a potential of +0.151 showed slight growth, as did 5 C at a potential of +0.153; 5 B at a potential of +0.158 showed no growth again.

At the higher potential levels the rate of acid production begins at a later time, and is less in amount, although these results show poor quantitative agreement.

Additional, untabulated experiments in which no accurate pH determinations were made except at the beginning and at the end of the experiment, show again that at an average potential of $+0.149$ (average deviation 0.004) maintained for thirteen hours, there was no increase in reducing power or appearance of turbidity whereas both turbidity and increase in reducing power appeared at lower levels (E_h $+0.121$, 0.138 , 0.122).

On the whole, our experiments indicate that at an E_h of $+0.150$ v. (pH about 6.6) growth cannot occur, or occurs only very slowly, although there is no apparent injury to the inoculum. At $+0.110$ there is no evidence of inhibition of growth. At intermediate levels ($+0.120$ to $+0.140$ v.) there seems to be some inhibition as evidenced by general slowing of the acid production.

The line dividing growth and no growth has, we believe, been roughly established at $+0.150$ v., ± 0.010 , at a pH of 6.5 to 6.6 . Lack of agreement between electrodes and fluctuation of potential levels combine to make a more exact figure difficult to determine.

Stopping growth and acid production by raising the potential level. A variety of experiments were done to determine how drastic a treatment is necessary to stop acid production and growth in cultures in which both have already been well established. These experiments were set up in the manner previously described and four are described in detail.

Experiment 1

Inoculum: 1 ml. brain-broth culture.

Initial pH level established: 6.55 .

Purified nitrogen was run in for 3 hours; then all gas was stopped. The potential, $+0.044$ at the time, continued falling. Acid production started in 4 hours. After $9\frac{1}{4}$ hours the potential was -0.005 and the pH, 5.88 . Air was blown in at this point, slowly at first, then more rapidly. After 55 minutes of very mild aeration the potential had risen to $+0.176$ and the pH dropped to 5.74 . There had been no effect on the rate of acid production. More vigorous air treatment for an hour brought the potential up to $+0.238$. The pH change during this hour amounted to only 0.02 pH. Then all gas was shut off. The pH

change started again immediately, although somewhat more slowly than before. During the hour after aeration the potential dropped very slowly down to $+0.200$ and the pH changed 0.07 unit, whereas in the hour preceding aeration it had changed 0.11 pH unit. The pH change continued subsequently while the potential slowly and steadily dropped past $+0.100$ v.

Experiment 2

Inoculum: 2 ml. 4-day brain-broth culture.

Gas mixture: nitrogen and air (in increasing proportion) to keep the potential at $+0.070$.

Initial pH level established: 6.65.

Acid production started in 6 hours. After 10 hours when ΔpH per hour was 0.10 and the pH was 6.45, the potential was gradually increased, slowly at first, then more rapidly until in 15 hours (from the start of the experiment) it had reached $+0.180$. The ΔpH per hour for the last hour only (E_h $+0.110$ to $+0.180$) fell off to 0.04. All gas was then shut off and the potential fell again while the acid production increased to its pre-aeration level.

Experiment 3

Inoculum: 1 ml. 5-day brain-broth culture.

Initial gas mixture used: purified nitrogen.

Initial pH level established: 6.5.

Acid production started in 7 hours. After 11 hours the potential was $+0.063$, the pH, 6.07. At this point air was substituted for the nitrogen. In one hour the potential had reached $+0.224$ and the pH had dropped to 5.96. There had as yet been no decrease in the rate of acid production. Within the next 10 hours, however, the pH dropped only to 5.71, indicating a marked decrease in the rate of acid production. During this time the potential rose slowly to $+0.243$ v. The air was then shut off, but the potential remained high and the rate of acid production did not increase. Ninety-five hours after the beginning of the experiment an attempt was made to start the culture again by passing in purified nitrogen. The potential fell to sufficiently low values but 24 hours of this treatment did not result in resumed activity.

Experiment 4

Inoculum: 1.5 ml. 5-day brain-broth culture.

Gas mixture: nitrogen and air (in increasing proportion) to maintain the potential at about $+0.115$.

Initial pH level established: 6.75.

Acid production seemed to start in 7 hours, but was slow (ΔpH per hour, 0.03). At the end of 13 hours when the pH was 6.55, air alone was bubbled through the culture for 17 hours. Then it was turned off. During this time acid production had ceased and the potential had reached +0.250. After the air had been turned off, the culture did not reestablish reducing conditions of its own accord and acid production did not start again. One hundred and twenty-one hours after the beginning of the experiment, pure nitrogen was bubbled through in an attempt to start the culture again. After about 36 hours of this treatment, growth started as evidenced by acid production.

The four experiments just described show that an actively growing culture of *Bacteroides vulgatus* can tolerate a temporary overdosage of oxygen, accompanied by a considerable rise in potential without apparent ill effects. There is a tendency for acid production to be slowed up at the higher potential levels, but cessation of aeration leads to an immediate reestablishment of pH change and lowering of potential, even though no nitrogen is bubbled through the medium. If the air treatment is continued for a longer time (10 to 20 hours), however, actual injury is sustained. The culture loses its ability to reestablish acid production and it is also unable of itself to develop a low potential; and renewed growth can only be secured after a long lag (more than 24 hours), if at all, by treatment with purified nitrogen and CO_2 . The results of this particular series of experiments do not, however, permit any conclusions about the limiting potentials where growth and acid production are just stopped. The only safe generalization that can be made is that potentials above +0.250 v. at pH's of about 5.6 to 6.4 stop the growth and acid production and reduce the viability of cultures already actively growing. The question of whether acid production and growth have distinct limiting potentials has not been answered.

Oxygen consumption

The rise in potential which occurred when air was administered in excessive doses to cultures of *Bacteroides vulgatus*, and the loss in reducing power which followed if this oxygen administration

was continued for a sufficient length of time, are probably both associated with an actual oxidation occurring in the culture. To test this point oxygen uptake was measured by means of the Warburg technique.

One-ml. samples of the supernatant liquid (stirred up) from 2-day brain-broth cultures were measured into Warburg manometer vessels. CO_2 was absorbed by $\text{N}/2 \text{ NaOH}$ placed in the cen-

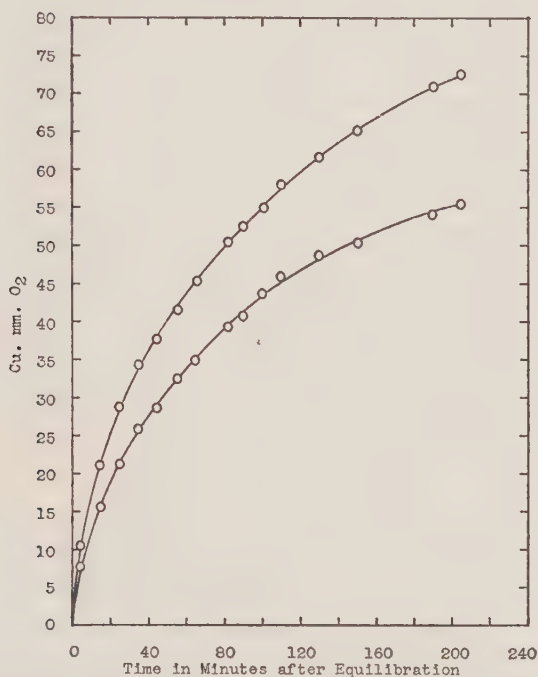


FIG. 6. O_2 CONSUMPTION OF 1 CC. OF 48-HOUR BRAIN-BROTH CULTURES OF *BACTERIODES VULGATUS*

tral cup. Figure 6 shows typical results obtained at 37°C . The oxygen consumption starts at a relatively high rate, averaging about 40 cm. for the first hour, but falls off steadily and rapidly. The uninoculated medium has a negligible oxygen consumption.

An attempt was made to measure the rate of oxygen consumption of the organisms growing on glucose-peptone medium, but the values obtained averaged only 2 or 3 cmm. per hour. This was attributed to the fact that the number of organisms per milli-

liter was small, as well as to the probability that the major part of the oxygen consumption probably occurred during the period necessary for equilibration of the manometers (15 minutes).

Peroxide production

Since so much attention has been given to peroxide production by bacteria (McLeod, 1931), it was thought interesting to test for the production of this compound by *Bacteroides vulgatus*.

The benzidine test of Avery and Morgan (1924) was used. Three-day cultures of the organism on Eggerth's synthetic medium, on buffered, 1 per cent glucose nutrient broth, and on the usual brain broth were prepared. These cultures were spread out in shallow layers in Petri dishes at 38°C. and tested at hourly intervals for 8 hours. All tests for peroxide were negative.

Since *Bacteroides vulgatus* forms no spores, its destruction by any peroxide produced would be thought to be more complete than that of the spore-forming anaerobes. Colonies of this organism on cystine-glucose nutrient agar contained viable organisms after 48 hours exposure to the air. The supposedly strong lethal effect of oxygen is not apparent. Obviously our results do not support the peroxide theory.

DISCUSSION

Comparison of our results with those of other investigators

It is interesting to note that the potential limit which we have determined for *Bacteroides vulgatus* agrees quite well with that obtained by Knight and Fildes, 1930, for *Clostridium tetani*. We adopted their method of poisoning potentials and used a medium essentially similar to theirs except for the addition of glucose and the omission of a buffer. We, however, measured the limitation of growth of an active inoculum of a non-spore-forming anaerobe. They measured the limitation of germination of washed spores. The difference between our value, +0.140 v. at pH 6.6 and their value, +0.110 v. at pH 7.0 to 7.65, for the level which just allows growth can easily be accounted for by the limitation of the accuracy of the method to 10 or 15 millivolts and by the possible variation of potential with pH. In fact, Knight and Fildes actually present some evidence that the limiting potential rises

with a fall in pH. The agreement between limits determined for *Clostridium tetani* and for *Bacteroides vulgatus* suggests that the mechanism of anaerobiosis may be similar in the two organisms.

Wide discrepancies exist, however, between our results and figures for limiting potentials determined by other investigators. All other workers have made potential measurements with dyes or with a combination of electrodes and dyes in media where little provision was made for poisoning potentials at definite levels for more than a restricted period of time. This last condition makes it seem probable that limits so determined would be too low. Knight and Fildes point this out in explaining the difference between their limit and one previously determined by Fildes (1929), who used a dye method and obtained a value which was 0.1 v. lower than that later established. Furthermore, the possibility exists that the limiting potential in the presence of an autoxidizable dye may not be the same as that in the absence of the dye.

Some investigators have followed another method of approach by attempting to separate the two factors, oxygen tension and potential. Now, in our experiments, a definite potential was maintained by controlling the oxygen tension. Limiting conditions were found to exist in the form of a definite E_h level, whereas the oxygen tension which the culture could tolerate varied widely with the state of the culture. These results indicate that oxygen tension controls the growth of this organism only insofar as it influences the potential. Knaysi and Dutky (1936), on the other hand, claim that the high potentials (+0.300 v.) associated with high oxygen tensions are more inhibitory to a butanol-producing *Clostridium* than the high potentials (+0.336 v.) maintained by potassium ferrieyanide. Their organism was not a strict anaerobe and the differences on which they based their conclusions were small. It would be interesting to see such observations extended, with more permanent poisoning than can be obtained by a single addition of a small amount of an oxidizing agent. The work of Kligler and Guggenheim (1937) indicates that the growth of *Clostridium welchii* under aerobic conditions in the presence of ascorbic acid is related to the lowering of the potential and

not to the absorption of oxygen from the medium. Although we agree essentially with the nature of some of their conclusions we believe that more accurate methods than they employed should be applied to the problem before the results can be regarded as satisfactory evidence to support the point.

*Significance of a potential limit and its place in a theory
of anaerobiosis*

In a study of such a nature that a quantitative method yields results which cannot be directly interpreted, it is often well to stop and ask whether the results have any meaning at all. Their first claim to significance lies in their reproducibility. It is of some importance in other words that a limit has actually been found to exist; or, that the appearance of life phenomena can be correlated with the relatively well-defined magnitude of a certain physical property of the culture.

Having established the fact that a potential limit exists, we face the question: just how is an electrode potential of a particular magnitude to be interpreted in these experiments? Does it represent the state of a particular reversible system? Does it represent the effective oxygen tension of the culture? Or is it a sort of grand average of several sluggish systems in only partial equilibrium with each other? Unfortunately, none of these questions can at present be answered with any certainty, and it would be pointless to add to the numerous discussions of these questions which have already appeared in the literature.

Under the circumstances, we have considered it permissible and desirable to set up a hypothesis which explains and is in full accord with all the known facts, and is, furthermore, amenable to experimental verification. This hypothesis has been formulated for the experimental conditions which we have employed as follows:

1. The potential measured in the *Bacteroides* cultures indicates the state of a reversible oxidation-reduction system.
2. Oxygen affects the potential by oxidizing this system (directly or indirectly).
3. The oxidizing action of oxygen is counteracted by reducing substances present in the culture which tend to reduce the reversi-

ble system. These reducing substances are derived from substrate molecules of high energy content which have been activated by enzymes (the dehydrogenases) of the cell.

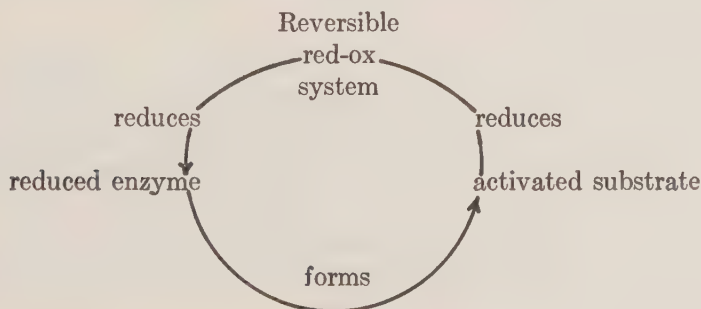
4. The enzyme system which activates the substrate is active only in the reduced form. It is maintained in this form by the reducing action of the activated substrate mediated by the particular reversible system which determines the electrode potential.

Then, at a potential of about +0.150 v. at pH 6.6, the reversible system can maintain the enzyme in a sufficiently active state to produce just enough reducing substances to counteract the effect of oxygen. At +0.140 v. and below, the reversible system keeps the enzyme sufficiently reduced to produce more than enough reducing substances to counteract the effect of oxygen. Consequently the reducing power of the culture increases and the orderly disposition of the extra energy available leads to growth. Above +0.150 v. the reducing substances produced cannot quite keep up with the oxygen. The enzyme is completely inactivated by oxidation and may eventually be destroyed.

This hypothesis deals with the following more or less complex components of the bacterial culture:

1. Activated substrate.
2. An enzyme system which activates the substrate and which is active only in the reduced form.
3. A reversible oxidation-reduction system directly or indirectly oxidized by oxygen and in real or partial equilibrium with the enzyme system and with the activated substrate.

The following diagram indicates the relations which are thought to exist among these components:



When oxygen is admitted, it oxidizes the oxidation-reduction system and thereby causes an oxidation of the enzyme, preventing the further formation of reducing substances.

This representation is naturally highly schematic. The components (1), (2), and (3), listed may each be simple or complex, containing one or many compounds. The enzyme itself may be directly susceptible to oxygen or may undergo some spontaneous dismutation which is reversed by reduction. A single arrow in the diagram may indicate a whole series of intermediate steps.

The scheme proposed, however, is the simplest possible formulation of an essential mechanism which may be operating to cause anaerobiosis. The main ideas it embodies are that oxygen interferes with growth because it inhibits, by oxidation, one or several enzyme systems which bring about the activation of the substrate molecules; and that the potential limit as determined by Knight's method, is an indirect measure of the state of reduction of the enzyme system.

The general point of view developed in this hypothesis is by no means new. Quastel and Stephenson (1926) suggested that oxidation-reduction potential was a limiting factor in the growth of anaerobes insofar as it was a measure of the concentration of sulfhydryl and possibly other reducing groups. A quotation from their paper will illustrate their idea:

The problem resolves itself into one of relative velocities; if the velocity of oxidation of the $-SH$ be greater than the rate of its formation, then proliferation cannot occur; if proliferation has already commenced by reason of the initial presence of a quantity of $-SH$, then proliferation will continue as long as the minimal concentration of $-SH$ is maintained.

Extensive discussions of the relationship of E_h to anaerobiosis have been published by Hewitt (1936), Wurmser (1930), and Clark (1924, 1934). Wurmser (p. 341) suggested that in anaerobiosis as well as in the Pasteur effect, the effect of oxygen might be related to the inactivation of a catalyst.

Broh-Kahn and Mirsky (1938) have suggested that oxygen may form some loose chemical complex with the anaerobic system of

obligate anaerobes and thus remove it from activity. They reject the ideas that oxygen acts by oxidation and that a potential limit has any significance because they claim that the rise in potential observed when oxygen is added to an anaerobic culture is the result of activation of the gas by the electrode. In the absence of an electrode they claim that oxygen is chemically inert since strict anaerobes contain no catalysts for the activation of oxygen. But we have shown that fresh cultures of *Bacteroides vulgatus* consume oxygen actively. A further study of oxygen consumption by strict anaerobes will undoubtedly throw further light on the problem.

It is not, of course, necessary to assume that all the oxygen consumed was used to oxidize the enzyme. The interactions postulated between oxidation-reduction system, enzyme, and substrate might well lead to a considerable oxidation of the substrate before the enzyme was completely inactivated.

Furthermore, the extreme lability of the enzymes of *Clostridium sporogenes* to oxygen, as reported by Stickland (1934) lends support to the hypothesis that these enzymes are peculiarly susceptible to oxidation. This susceptibility of some anaerobic enzymes to oxygen may be a general phenomenon. Hoogerheide and Kocholaty (1938) found that even traces of air diminished hydrogen absorption by amino acids in the presence of *Clostridium sporogenes*. Of interest in this connection are the lack of appearance of hydrogenlyase in cultures grown aerobically; and the host of investigations on the association of the activity of a very wide variety of enzymes with the presence within the enzyme molecule of a reduced sulfur group (Bersin, 1935; Hopkins and Morgan, 1938; Hellerman, 1937).

SUMMARY

1. The course of potential and pH changes in mixed cultures of *Bacteroides vulgatus* and *Alcaligenes fecalis* on glucose nutrient broth and on a synthetic medium were studied; but attempts to determine a positive potential limiting the growth of the *Bacteroides vulgatus* were unsuccessful by this method.

2. Potentials of cultures of *Bacteroides vulgatus* on glucose

nutrient broth were controlled by means of a mixed stream of nitrogen and oxygen and by this method the positive potential just limiting growth was found to be $+0.150$ v. at pH 6.6.

3. CO_2 was found to be essential for the growth of *Bacteroides vulgatus*.

4. Glucose brain-broth cultures of *Bacteroides vulgatus* were found to show a marked oxygen consumption.

5. A theory of anaerobiosis has been proposed; and the relation of this theory to a potential limit for anaerobic growth and to oxygen consumption by anaerobes has been discussed.

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